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Fate Restriction in the Growing and Regenerating Zebrafish Fin

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SUMMARY

We use transposon-based clonal analysis to identify the lineage classes that make the adult zebrafish caudal fin. We identify nine distinct lineage classes, including epidermis, melanocyte/xanthophore, iridophore, intraray glia, lateral line, osteoblast, dermal fibroblast, vascular endothelium, and resident blood. These lineage classes argue for distinct progenitors, or organ founding stem cells (FSCs), for each lineage, which retain fate restriction throughout growth of the fin. Thus, distinct FSCs exist for the four neuroectoderm lineages, and dermal fibroblasts are not progenitors for fin ray osteoblasts; however, artery and vein cells derive from a shared lineage in the fin. Transdifferentiation of cells or lineages in the regeneration blastema is often postulated. However, our studies of single progenitors or FSCs reveal no transfating or transdifferentiation between these lineages in the regenerating fin. This result shows that, the same as in growth, lineages retain fate restriction when passed through the regeneration blastema.

INTRODUCTION

In regenerating limbs or fins of salamanders or fish, following amputation, a blastema of dedifferentiated and proliferating cells is recruited from the stump, which then grows out to replace the missing portion of the limb or fin. Whether cells in the blastema transdifferentiate to other fates during subsequent outgrowth of the limb is unclear (Akimenko et al., 2003; Tal et al., 2010; Tamura et al., 2009), though several studies have shown some fate restriction in *Xenopus* tail or salamander limb regeneration (Kragl et al., 2009; Gargioli and Slack, 2004; Hay and Fischman, 1961; Muneoka et al., 1986). The zebrafish fin provides a useful model to address questions of lineage. Like the embryo the zebrafish adult fin is largely transparent, with the exception of the pigmented melanocytes (black cells), xanthophores (yellow cells), and iridophores (shiny cells) (Hirata et al., 2005; Parichy et al., 2009) (Figure 1A). Histological analyses of a number of small teleost fins, including zebrafish fins, have shown that they are comprised of a relatively small number of cell types, including the three pigment cell types mentioned above, osteoblasts that synthesize the bone matrix (Akimenko et al., 2003; Marí-Beffa et al., 1996; Poss et al., 2003; Smith et al., 2008), dermal fibroblasts (Marí-Beffa et al., 1996; Montes et al., 1982), artery and vein endothelium (Becerra et al., 1983; Huang et al., 2009; Montes et al., 1982), nerves, including the lateral line system (between fin rays) (Ghysen and Dambly-Chaudiere, 2007; Marí-Beffa et al., 1996; Martorana et al., 2001; Poleo et al., 2001; Wada et al., 2008) and the intraray nerve comprised of sensory and motor nerve axons and associated glial cells (Becerra et al., 1983; Montes et al., 1982), skin epidermis (Marí-Beffa et al., 1996; Martorana et al., 2001; Poleo et al., 2001), and resident blood cells, including macrophages, plasma cells, and neutrophils (Hall et al., 2007; Zhao et al., 2008). Notably absent from the distal, or fin ray, portion of the fin are striated muscle and cartilage (Becerra et al., 1983; Marí-Beffa et al., 1996; Montes et al., 1982) (Figure 1).

The lineage relationship between the different cell types in the fin is largely unknown or postulated. One exception is the relationship of the pigment cells. Transposon-based clonal analysis shows that the melanocytes and xanthophores develop from one population of organ founding stem cells (FSCs), whereas the iridophore develops from a distinct population of organ FSCs (Tu and Johnson, 2010).

Other lineage relationships have been partially addressed in the regenerating tadpole tail, where spinal cord and notochord regenerated from the same labeled tissue (Gargioli and Slack, 2004), and also in salamander limb regeneration, where different labelings have been employed to show that muscle does not give rise to cartilage, and cartilage does not give rise to muscle (Kragl et al., 2009). However, limitations in specific labeling of each cell type have left the possibility of transdifferentiation open for some cell types. For instance, in salamander limb regeneration, labeled dermis contributes to labeled cartilage in the regenerate. One explanation for this result is that labeled, transplanted dermis includes cartilage precursors but also raises the possibility that dermal fibroblasts transdifferentiate in the blastema to form cartilage. Also, Kragl et al. (2009) showed that labeled Schwann cells do not transdifferentiate into cartilage. Transdifferentiation of glial cells into a more closely related cell type—the melanocyte—could not be assessed in those experiments because they were performed in a genetic background that lacked melanocytes.

In this study we took advantage of a lineage-tracing method (Tu and Johnson, 2010) (Figure 2A) to examine the lineage relationships between the different cell types in the fin, and the possibility of transdifferentiation in the regeneration blastema, by clonal analysis. Our results show that in the zebrafish fin, osteoblasts and dermal fibroblasts are unrelated lineages, artery and vein arise from the same progenitors, and neuroectodermal

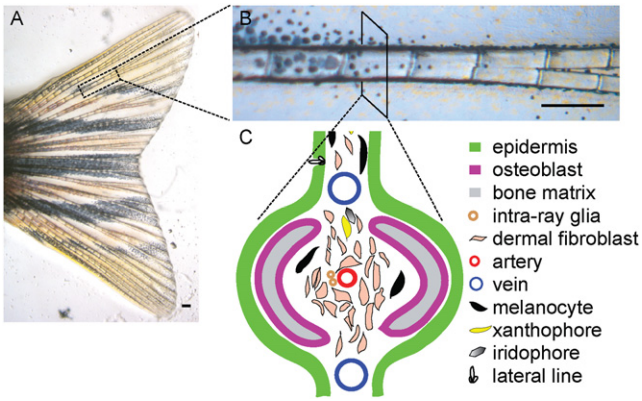


Figure 1. The Anatomy and Different Cell Types of the Zebrafish Caudal Fin

(A–C) The zebrafish adult caudal fin is almost transparent, except that it has some pigmented cells: the melanocytes (black cells), xanthophores (yellow cells), and iridophores (shiny cells) (A). The caudal fin is supported by 18 bony fin rays (B). Cross section of a single fin ray identifies at least ten different cell types. The organization of the different cell types in the fin ray is illustrated in (C). Scale bars, 0.2 mm.

cell types—including the two previously described pigment cell lineages (Tu and Johnson, 2010), intraray glia, and lateral line—each have independent origins in the fin primordium. Moreover, amputation and regeneration through clone-bearing fins showed that clones always recapitulated their fates in the regenerate, tending to rule out a role for transdifferentiation in the blastema in normal fin regeneration.

RESULTS AND DISCUSSION

Generation of Genetically Labeled Clones

To identify the constituent lineages of the adult fin, we injected one or two cell zebrafish embryos with a Tol2 lineage marker transposon carrying a ubiquitously expressed *Xenopus EF1α* promoter driving GFP expression (Figure 2A). Typically following injection, Tol2 integrates at approximately the 4000 cell stage (R. Tryon and S.L. J., unpublished data). Stable transgenics from this or similar constructs are typically partially downregulated in mature fins, then strongly upregulated in regenerates. Although the *EF1α* promoter is reported to be silenced in some adult tissues, it is expressed in virtually all cells of both normally grown fins and of regenerates, including in variegating stable lines (Burket et al., 2008; Thummel et al., 2006) (see Figure S1 available online), making this an appropriate lineage marker for our analysis.

When only one or relatively few cells of the blastula experience transposition, extensive cell movements during ensuing gastrulation result in highly mosaic embryos at 1 day postfertilization (dpf) (Figure 2A). The Tol2-generated clones are again bottlenecked in the fin primordium (between 2 and ~12 dpf), which results in the organ FSCs described here. We note that it remains possible that proliferation and subsequent fate segregation following the integration event in the gastrulation stage embryo could result in more than one labeled FSC in the fin primordium, generating more than one lineage class but that such events will be unlikely to show strong co-occurrence in the larger data set.

Co-Occurrence Analysis Identifies Nine Lineage Classes

From approximately 5500 injected zygotes, we identified approximately 3000 mosaic embryos at 1 dpf, 1175 of which survived to mature stages. Examination of these fish revealed 322 (27%) with GFP expression in the caudal fin. GFP expression was typically observed as mosaic labeling of one or more cell types in the fin. An example of a fin with more than one labeled cell type is shown in Figure 2B. Assuming a binomial distribution of clones in the fins suggests that perhaps 27% of our fin labelings were the result of multiple clones, and thus, finding two different labeled cell types in the same fin need not indicate that they arose from the same lineage or FSC. To correct for this, we first scored each fin for each labeled cell type, then assessed the frequency of co-occurrence, and the probability of chance association through polyclonal FSCs. We reasoned that cells that sometimes colabeled by chance should be randomly associated, whereas cells that colabeled because they belonged to the same lineage should always or almost always colabel.

Figure 2C shows our co-occurrence analysis matrix for GFP-labeled clones in a subset of 116 caudal fins that were more carefully examined for all GFP labelings. This analysis shows that in most pairwise comparisons, the lineage marker does not co-occur significantly between the two cell types. Two exceptions are the complete or near-complete co-occurrence of label in melanocytes and xanthophores or arteries and veins: 9 of 9 fins bearing labeled melanocytes also had labeled xanthophores in the same region of the fin (Tu and Johnson, 2010), and 21 of 22 fins with labeled arteries also had labeled veins. Moreover, vein labelings were in the same fin rays as those with labeled arteries. These results suggest that like the melanocyte and xanthophore that develop from the same FSC, the artery and vein also develop from the same organ founding cells, arguing against independent origins of fin ray arteries from dorsal aorta and fin ray veins from cardinal vein. We also saw a tendency for co-occurrence between the labeled melanocyte/xanthophore class with intraray glia, although the significance level of this association ($p = 0.016$) was greater than the 5% significance threshold when adjusted for multiple testing ($p < 0.0009$). This weak association, that 2 of 4 fins with labeled intraray glia also had labeled melanocytes and xanthophores, may reflect a common origin in the neural crest, rather than a later common origin in the fin primordium.

Our analysis also shows which cell types do not share a common precursor, or FSC, in the fin primordium and thus allows us to identify all of the lineage classes in the adult fin. In addition to the melanocyte/xanthophore lineage, the iridophore lineage (Tu and Johnson, 2010), and the artery/vein endothelium lineage (Figure 2H), we identify the intraray glia (Figure 2D), lateral line (Figure 2E), osteoblasts (Figure 2F), dermal fibroblasts (Figure 2G), skin epidermis (Figure 2I), and resident blood (Figures 2J and 2K) as discrete lineage classes. One prediction from the model that these are in fact distinct lineages is that we should observe fins with label solely in one of each lineage class. Indeed, in the 116 caudal fins examined, we observed 1 fin bearing only labeled melanocytes and xanthophores, 1 fin bearing only labeled iridophores, 1 fin bearing only labeled osteoblasts, 14 fins bearing only labeled dermal fibroblasts, 1 fin bearing only labeled artery/vein endothelium, 2 fins bearing

only labeled lateral line, and 11 fins bearing only labeled skin epithelium. These results confirm each of those cell types as discrete lineage classes.

We failed to find fins bearing only labeled intraray glia or resident blood. The notion that the intraray glia are a distinct lineage class is, instead, supported by the observation that we can identify fin rays with isolated intraray glia clones and lack other labeled cell types, such as that shown in Figure 2D. Thus, whereas there were 72 fin rays in 4 fins with labeled intraray glia, 11 of these rays had no other cell types labeled, tending to confirm the lineage class. We suspect that our failure to find intraray glia-only fins reflects an ascertainment bias in identifying this lineage class in our primary screen. This labeling is identified during our more careful scrutiny during our secondary examination of fins identified bearing other clones. Our failure to find resident blood-only fins may reflect a similar ascertainment bias.

We conclude that the adult caudal fins are generated from nine distinct lineage classes. Most of these classes contain one or very few cell types. Thus, the melanocyte/xanthophore lineage class consists only of those two types, the iridophore lineage class consists only of iridophores, the endothelium class consists only of fin ray artery and vein cells, and the lateral line class consists of the hair cells and glial cells of the lateral line. We identify a skin epidermis lineage class but have not explored whether this class consists of multiple cell types or, possibly, distinct lineages. The osteoblast lineage consists of the osteoblasts that surround and secrete the bone matrix. In some cases we have observed somewhat more intense GFP expression in the joint cells separating the bone segments than in the neighboring osteoblasts (data not shown). Because we never observe labeled joints without associated osteoblasts, we conclude that the joint cells are part of the osteoblast lineage we identify here. Our analysis of the dermal fibroblast lineage suggests a single-cell type: the loose mesenchymal cells that fill the space between the fin hemirays. We suspect a second cell type in this lineage, the smooth muscle pericyte associated with the fin ray artery (Bayliss et al., 2006), although examination of confocal images of dermal fibroblast clones fails to suggest intimate association of any of the labeled cells with the artery. Our finding of distinct lineage classes for the osteoblasts and the dermal fibroblasts tends to dispel the commonly held assumption that the fin ray osteoblasts develop as a condensation from the fin ray dermal fibroblasts (Haas, 1962; Johnson and Bennett, 1999). We also identified the intraray glia lineage class that is associated with the intraray nerve. These nerves are still poorly studied and may contain several distinct types of glial or support cells that with similar experiments as those reported here and better markers might further subdivide this class into more than one lineage. Resident blood also is several cell types, including macrophages, LysC-expressing neutrophils (Hall et al., 2007), and possibly antibody secreting B or plasma cells (Zhao et al., 2008).

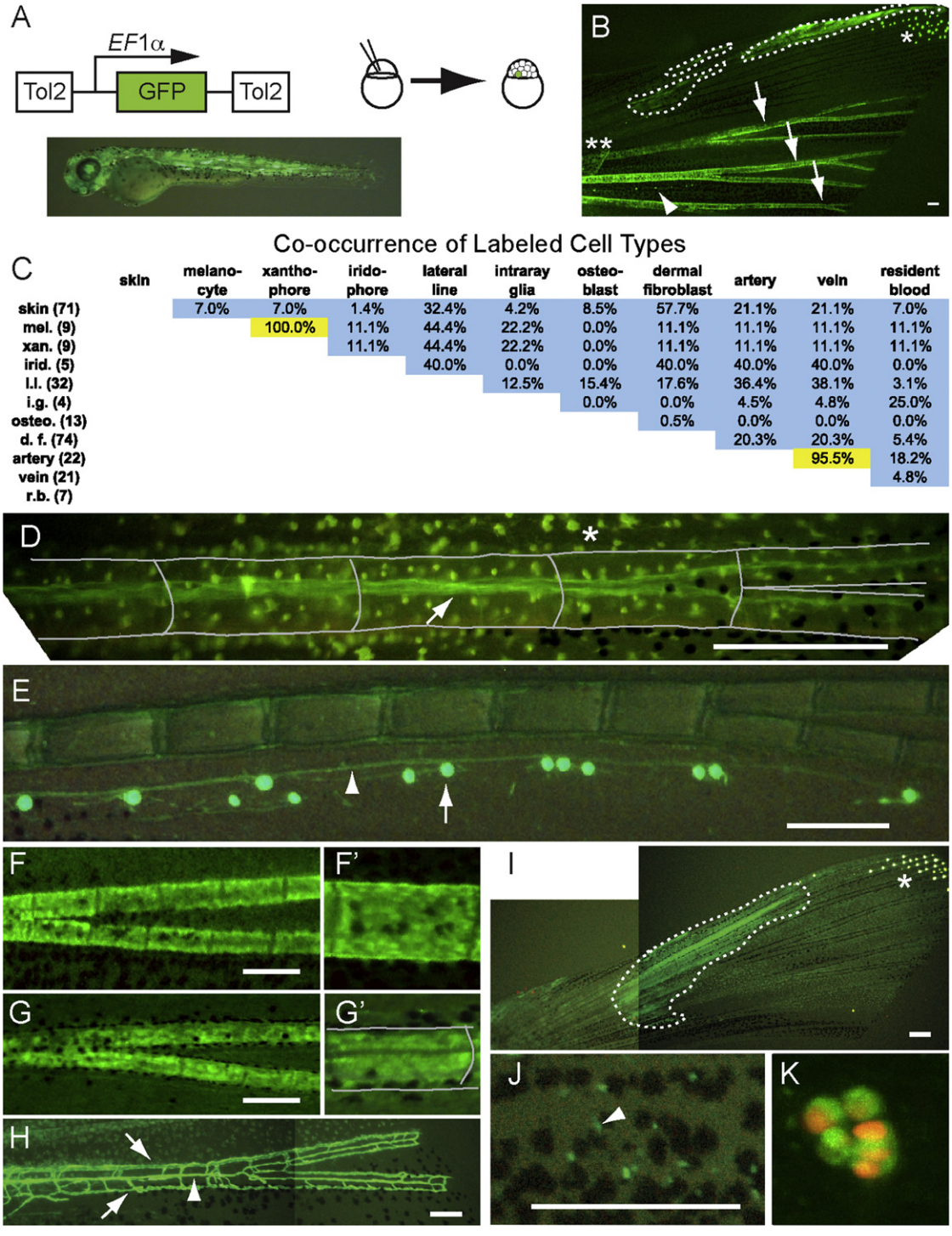
Growth Patterns of the Different Lineages

In general these clone classes extend distally along the proximodistal axis of the fin, consistent with clone growth at the distal margin of the fin (Goldsmith et al., 2003, 2006; Haas, 1962; Tu and Johnson, 2010). Most clones are also typically a few fin rays in width, and never contribute to all the fin rays, suggesting that more than one FSC of each class colonizes the fin primordium. Also, we often see mosaicism within the region occupied by pigment cell, osteoblast, dermal fibroblast, and vascular clones, suggesting that daughters of more than one FSC contribute to any particular region or ray.

Two lineage classes, epidermis and resident blood cells, show different patterns. Epidermal clones are found as patches of labeled cells, often with both proximal and distal boundaries in the interior of the fin (Figure 2I), suggesting that the epidermis grows by radial expansion, rather than proximodistally. Labeled resident blood cells are found throughout the entire fin (Figure 2J), and also associated with circulating blood cells (data not shown), suggesting that they may populate the fin by blood circulation instead of a founding event in the fin primordium. One example of labeled resident blood cells showed that the GFP^+ cells are only a subset of all the neutral red positive macrophages (data not shown) (Ellett et al., 2010; Winckler, 1974), suggesting that more than one resident blood progenitor cell contributes to the fin resident blood cells.

Lineage Restriction during Fin Regeneration

The finding that each lineage class is limited to a small number of cell types indicates that fate is highly restricted during normal development and growth of the fin. To further examine fate restrictions, we investigated how each of these clones regenerated following passage through the regeneration blastema. Like salamander the zebrafish regeneration blastema is an apparently homogeneous group of dedifferentiated cells that are recruited from the tissues of the stump (Haas, 1962; Hay, 1958). This regeneration proceeds first by cell division immediately proximal to the amputation plane of osteoblasts and dermal fibroblasts (and possibly other mesenchymal cell types) beginning at ~24 hr postamputation (Johnson and Bennett, 1999; Nechiporuk and Keating, 2002; Poleo et al., 2001). Daughters of these divisions then migrate or are displaced distally to form a homogeneous-appearing blastema by 2–3 days postamputation. Transplants of labeled tissues in *Xenopus* tail or salamander limb regeneration have tended to rule out transfating or transdifferentiation between major tissue classes, for instance between ectoderm and mesoderm, but have left open the possibility of transfating between cell types of the connective tissue, or cells of neural crest origin (Gargioli and Slack, 2004; Kragl et al., 2009; Muneoka et al., 1986). We reasoned that our finding of discrete lineages arising from single FSCs could allow us more precise resolution of this question. Accordingly, we challenged fins bearing each type of clone (except iridophore clones) to regenerate for 2 weeks, then examined whether the labeled cells in the regenerate were the same or different from in the stump. In all cases we found that label in the regenerate precisely matched the labeled cell type in the stump. Thus, melanocyte/xanthophore clones only regenerated labeled melanocytes and xanthophores and never regenerated labeled cells of the other neuroectodermal derivates—iridophores, intraray glia, or lateral line (Figure 3A). Similarly, intraray glia clones only contributed label to the regenerating nerve upon regeneration, and never resulted in labeled pigment cells or lateral line (Figure 3B), and lateral line clones never regenerated labeled intraray glia or pigment cells



	skin	melano-cyte	xantho-phore	irido-phore	lateral line	intraray glia	osteo-blast	dermal fibroblast	artery	vein	resident blood
skin (71)		7.0%	7.0%	1.4%	32.4%	4.2%	8.5%	57.7%	21.1%	21.1%	7.0%
mel. (9)			100.0%	11.1%	44.4%	22.2%	0.0%	11.1%	11.1%	11.1%	11.1%
xan. (9)				11.1%	44.4%	22.2%	0.0%	11.1%	11.1%	11.1%	11.1%
irid. (5)					40.0%	0.0%	0.0%	40.0%	40.0%	40.0%	0.0%
l.l. (32)						12.5%	15.4%	17.6%	36.4%	38.1%	3.1%
i.g. (4)							0.0%	0.0%	4.5%	4.8%	25.0%
osteo. (13)								0.5%	0.0%	0.0%	0.0%
d. f. (74)									20.3%	20.3%	5.4%
artery (22)										95.5%	18.2%
vein (21)											4.8%
r.b. (7)											

Figure 2. Clonal Analysis Identifies Nine Distinct Lineage Classes in the Zebrafish Fin

(A) A Tol2 transposon lineage marker was injected into one or two cell embryos as previously described (Tu and Johnson, 2010). A highly mosaic embryo is indicative of transposon integration.

(B) An example of caudal fin (dorsal half) with mosaic GFP expression. This fin has three types of labelings: three patches of labeled epidermis (dotted line), labeled osteoblasts in three fin rays (arrows), and a string of labeled lateral line neuromasts (arrowhead). Single asterisk indicates reflection from white cells (Johnson et al., 1995) at the tip of the dorsal lobe. Double asterisks indicate labeled scale (not part of the fin) on the tail of the fish.

(C) Analysis of co-occurrence of GFP-labeled cell types. The first column lists the different cell types in the fin, with the number of fins carrying the labeled cell type in the data set of 116 mosaic caudal fins. Numbers shown in matrix cells indicate the co-occurrence of each cell type as percentage of total number of fins with labeled cell type in the first column. For example, within the 71 fins carrying labeled epidermis, 7.0% of them also carried labeled melanocytes, which is the observed co-occurrence between the two cell types. Comparing that to the expected co-occurrence (predicted by multiplying the percentage of each cell type)

(Figure 3C). Thus, we saw no evidence for transfating among the four neuroectodermal lineage classes. We also found no evidence for transfating among the three mesodermal lineage classes, osteoblasts (Figures 3E–3G), dermal fibroblasts (Figures 3H–3J), and vascular endothelium (Figure 3K), during regeneration. We note especially that it might be difficult to detect just a few labeled dermal fibroblasts in medial positions within a fin ray bearing an osteoblast clone, or alternatively, a few labeled osteoblasts surrounding the bone matrix when the dermal fibroblasts are labeled. Confocal image analysis of regenerates of five osteoblast clones and seven dermal fibroblast clones (each containing hundreds of labeled cells) revealed no labeling in the wrong positions that might be masked in whole-mount observations. This result suggests that if dermal fibroblast and osteoblast lineages interconvert, such instances are rare in the zebrafish fin. Likewise, epidermal clones only contributed labeled skin to the regenerate (Figure 3D).

We were also interested in the possible role for circulating stem cells contributing to the regenerate. We amputated fins from 150 fish with labeled clones in their body, but not their fins, indicative of at least one integration event in the fish, and allowed them to regenerate for 2 weeks. In no case did they result in labeled cells in the regenerate, tending to rule out a contribution by circulating stem cells to the fin regenerate. One possible exception is that when fins with labeled resident blood were amputated, labeled resident blood was also found in the regenerate. Whether these cells came from the stump or from the circulation was not investigated.

We note that we have not excluded roles for transdifferentiation within each lineage class. Thus, the supporting glia of the lateral line can convert to mechanosensory hair cells (Ghysen and Dambly-Chaudiere, 2007), and it remains possible that arteries and veins also convert to the reciprocal fate, either in growth or in resolution of the regeneration plexus (Huang et al., 2009). Development of techniques that generate labeling at later stages, such as inducing genetic labeling with heat shock (Collins et al., 2010), may help resolve whether transdifferentiation can occur within a lineage class.

We also note that our finding of no transdifferentiation between lineage classes in normal growth or regeneration does not preclude that it could happen in other circumstances. One possibility is that the presence of cells of one lineage acts to prevent other lineages from generating those cell fates. This could be assessed by developing conditional techniques (Curado et al., 2007) to kill an entire lineage class including its stem cells and then assessing whether other lineages could replace it during growth or regeneration. Alternatively, it may be interesting to use this model of transposon-labeled clones to deliver ectopic gene expression in search of mechanisms that abrogate the strict fate restriction described here.

Spatial Restriction in the Blastema

Our regeneration analysis showed that clones retain fate and relative position between the stump and the differentiated regenerate. This led us to ask whether the clone also retains spatial restrictions within the early blastema, or whether cells from different lineages become mixed in the blastema, and then sort out during differentiation. To examine this, we took confocal images through 3–4 day regenerates bearing osteoblast or dermal fibroblast clones. These fins were stained with antibodies against GFP to reveal the clone and DAPI to reveal tissue architecture. Longitudinal sections through these images reveal that the dermal fibroblast clones remain medial within the blastema, similar to their medial position in the differentiated fin ray. An exception to this behavior is that at the distal end of the blastema of the dermal fibroblast clone, the labeled fibroblasts are not excluded from the lateral domain of the blastema (Figure 4A). This region corresponds to the distal-most blastema (DMB) (Nechiporuk and Keating, 2002), a region of the blastema that withdraws from the cell cycle between 3 and 4 days. Thus, we identify the dermal fibroblast lineages as major contributor to the DMB. In contrast, osteoblast clones are restricted to lateral positions in the blastema, similar to their position in the differentiated fin ray (Figure 4B). Additionally, unlike the dermal fibroblast clones, the osteoblast clones are excluded from the DMB. This behavior for the osteoblast clones is identical to that observed by staining fin regenerates with the monoclonal antibody ZNS5 (Johnson and Weston, 1995), which stains differentiated and undifferentiated osteoblasts. Thus, the two major components of the blastema, the dermal fibroblast lineage and the osteoblast lineage, retain their medial-lateral spatial restrictions within the blastema, except for the DMB, which may be entirely from the dermal fibroblast lineage. Whether spatial restriction influences the observed fate restrictions, fate restrictions cause the observed spatial restriction, or

by using a chi-square test yields a p value indicating that the difference is not statistically significant (denoted by light-blue shading); thus, the epidermis and melanocytes are not significantly associated and most likely arise from different FSCs. The same chi-square test was carried out for all pairwise combinations of any two cell types in the fin. p values were calculated to determine whether the associations were significant. Light blue indicates nonsignificant association, whereas yellow shows significant association. The multiple testing-adjusted threshold for 95% significance is p = 0.0009.

(D) Labeled intraray glia clone. Arrow points to the intraray nerve with labeled glia. Asterisk indicates strong autofluorescence from the xanthophores. Thin gray line outlines the fin ray and the joints.

(E–G) Labeled lateral line clone (E). Arrow points to a GFP^+ neuromast, and arrowhead points to GFP^+ interneuromast glia. Notice lateral line resides in the space between fin rays. Osteoblast clone (F) looks like a sheet of cells covering the mineralized bone matrix. Dermal fibroblast clone (G) appears more punctate, and encased between the hemirays, and GFP signal is excluded from where the artery lies. Notice that labeled osteoblast clone and dermal fibroblast clone have clearly different appearances in high magnification (F′ and G′).

(H) Vascular clone containing both artery (arrowhead) and vein (arrows).

(I) Labeled epidermis (skin) clone (outlined by dotted line). Note that this epidermis clone has both proximal and distal boundaries. Asterisk indicates reflection from white cells.

(J) Labeled resident blood cells (arrowhead).

(K) Neutral red (macrophage marker) staining of resident blood clone (Ellett et al., 2010; Winckler, 1974). Green indicates GFP from live fish. Red shows neutral red staining. Scale bars, 0.2 mm. See also Figure S1.

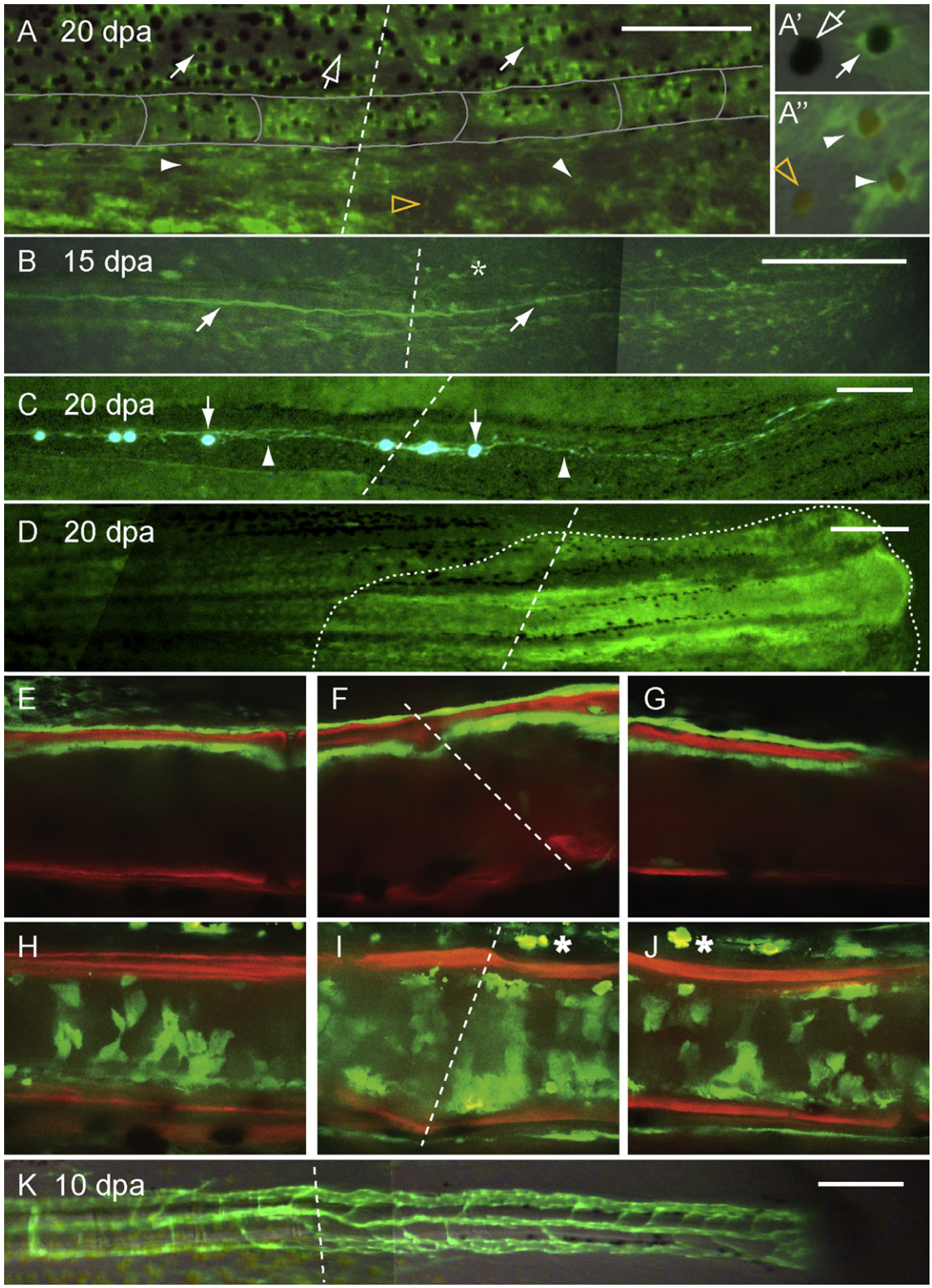


Figure 3. Lineages Do Not Transfate in the Regenerating Zebrafish Fin

(A) Labeled melanocyte/xanthophore clone, shown at 20 days postamputation (dpa), only regenerated melanocytes and xanthophores. Arrows and arrowheads point out the labeled melanocytes and xanthophores proximal (left) and distal (right) to the amputation plane (dashed line), respectively. The empty white arrow points to an unlabeled melanocyte, whereas the empty yellow arrowhead points to an unlabeled xanthophore. A GFP-labeled melanocyte appears as a black circle surrounded by a green halo (A′), whereas a GFP-labeled xanthophore appears as a yellow circle surrounded by a green halo (A″). Light-gray line outlines the fin ray.

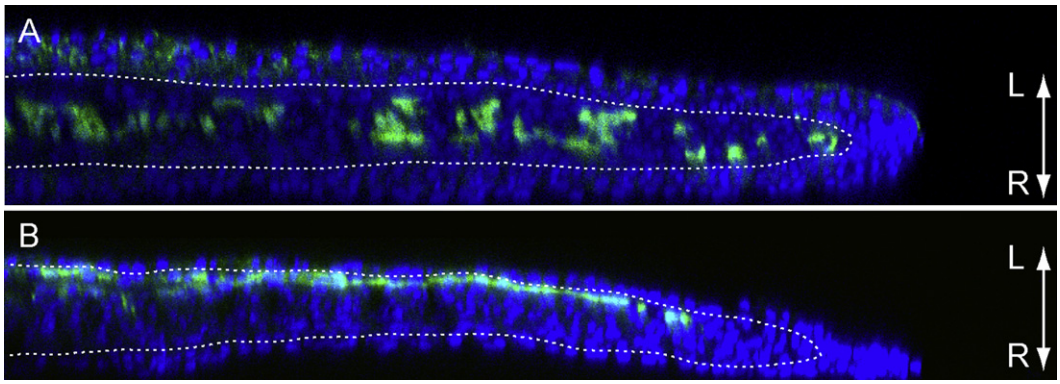


Figure 4. Confocal Microscopy Shows Spatial Restriction of Dermal Fibroblasts and Osteoblast in the Regeneration Blastema
(A) Dermal fibroblast clone in 4 days postamputation (dpa) regeneration blastema.
(B) Osteoblast clone in 4 dpa regeneration blastema. Dotted line outlines the basement membrane. Blue shows DAPI stain and green illustrates GFP (immunohistochemistry). L and R denote left and right sides of fish, respectively.

whether the two phenomena are independently regulated remains unclear.

EXPERIMENTAL PROCEDURES

Screen for Fins with Mosaic GFP Expression

We generated animals with mosaic GFP expression as previously described (Tu and Johnson, 2010). A primary screen was carried out by looking for GFP expression in 1175 adult zebrafish caudal fins with low magnification, identifying 322 caudal fins with labeled cells. Following this a secondary screen more closely scrutinized a subset of 116 randomly chosen labeled caudal fins for every GFP-labeled cell type under higher magnification. The labeled cell types and the positional information of each labeled cell types were recorded for each of the 116 fish.

Microscopy

Whole-mount epifluorescence images of clones were captured using ProgRes C14 software on an Olympus SZX12 dissection microscope, with X-Cite Series120 light source, as previously described. Amputations were carried out as previously described (Johnson and Bennett, 1999).

Confocal images of live fish and immunohistochemistry-treated fin samples were captured with an EMCCD camera attached to a Zeiss AxioObserver Z1 microscope, C-Aprochromat 40×/1.2 W Korr UV-VIS-IR objective, with a PerkinElmer UltraVIEW VoX laser-scanning disk confocal system. Bone matrix stained with alizarin red and macrophages stained with neutral red were both observed in the red channel.

Vital Dye Staining and Immunohistochemistry

To visualize the bone matrix in contrast to the GFP-labeled osteoblasts and fibroblasts (Figures 3E–3J), we soaked live fish-bearing clones in 200 μg/ml alizarin red (Sigma A5533) (Javidan and Schilling, 2004) in system water for 10 min, followed by rinsing out the fish three times in fresh water. We used neutral red as a marker for macrophages (Ellett et al., 2010; Winckler, 1974). Adult fish were in 25 μM of neutral red (Sigma N-6634) in system water for 1 hr, followed by several rinses with system water.

Immunostaining with rabbit anti-GFP (Torrey Pines; TP401, 1:1000) and donkey anti-rabbit Alexa Fluor 488 (Invitrogen; A 21206, 1:300) was performed to detect GFP-labeled cells in fixed fin samples. Samples were incubated in mounting medium with DAPI overnight to visualize tissue architecture.

All images have their levels adjusted by using Photoshop software to increase the contrast of the GFP labelings.

Statistical Analysis

We performed a chi-square test to find significant association of labelings in different cell types. The expected labeling occurrence in two cell types was calculated by multiplying the labeling frequency of one type of cells to labeling frequency of the other. The observed co-occurrence was calculated as percentage of fish having both types of labeling in the fin. We carried out Bonferroni correction for multiple testing.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at doi:10.1016/j.devcel.2011.04.013.

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(B) Intraray glia clone (arrows) only regenerates label in the intraray nerve. Asterisk denotes autofluorescence from xanthophores.
(C) A lateral line clone regeneration at 20 dpa. Arrows and arrowheads point to the lateral line neuromasts and glia proximal (left) and distal (right) to the amputation plane, respectively.
(D) Epidermis (skin) clone only regenerated epidermis.
(E–J) Confocal images (sagittal view) of a regenerating osteoblast clone (E–G), and a dermal fibroblast clone (H–J) at three positions: proximal to amputation plane (E and H), at the amputation plane (F and I), and distal to the amputation plane (G and J). Note that both clones are mosaic in the fin ray, with the osteoblast clone forming a continuous sheet immediately adjacent to medial and lateral surfaces of the bone matrix, in this case labeling the dorsal (top) of the ray, whereas the dermal fibroblast clone labels a population of loose mesenchymal cells in the interior of the fin ray. Dermal fibroblast clones are typically intermixed with unlabeled dermal fibroblasts throughout the intraray space. Red shows alizarin red staining of the bone matrix. Asterisk denotes autofluorescence from xanthophores.
(K) Whole-mount stereoscope view of regenerating vasculature clone; note that only vasculature carries label in the regenerate. Green shows GFP from labeled clone in live fish. Dashed lines indicate amputation plane. Scale bars, 0.2 mm. See also Figure S1.

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